Open Stomata 1 Kinase is Essential for Yeast Elicitor-Induced Stomatal Closure in Arabidopsis

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We recently demonstrated that yeast elicitor (YEL)-induced stomatal closure requires a Ca^{2+}-dependent kinase, CPK6. A Ca^{2+}-independent kinase, Open Stomata 1 (OST1), is involved in stomatal closure induced by various stimuli including ABA. In the present study, we investigated the role of OST1 in YEL-induced stomatal closure in Arabidopsis using a knock-out mutant, *ost1-3*, and a kinase-deficient mutant, *ost1-2*. YEL did not induce stomatal closure or activation of guard cell S-type anion channels in the *ost1* mutants unlike in wild-type plants. However, YEL did not increase OST1 kinase activity in wild-type guard cells. The YEL-induced stomatal closure and activation of S-type anion channels were also impaired in a gain-of-function mutant of a clade A type 2C protein phosphatase (ABA INSENSITIVE 1), *abi1-1C*. In the *ost1* mutants like in the wild type, YEL induced H_2O_2 accumulation, activation of non-selective Ca^{2+}-permeable cation (I_{Ca}) channels and transient elevations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in guard cells. These results suggest that OST1 kinase is essential for stomatal closure and activation of S-type anion channels induced by YEL and that OST1 is not involved in H_2O_2 accumulation, I_{Ca} channel activation or $[Ca^{2+}]_{cyt}$ elevations in guard cells induced by YEL.

Keywords: Arabidopsis • Calcium signaling • Guard cell • Open Stomata 1 • S-type anion channel • Yeast elicitor.

Abbreviations: ABI1, ABA INSENSITIVE 1; $[Ca^{2+}]_{cyt}$, cytosolic free Ca^{2+} concentration; CDPK, calcium-dependent protein kinase; CFP, cyan fluorescent protein; DTT, dithiothreitol; GCP, guard cell protoplast; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; I_{Ca} channel, non-selective Ca^{2+}-permeable cation channel; OST1, Open Stomata 1; PP2C, type 2C protein phosphatase; *RD29B*, *Responsive to Desiccation 29B*; SnRK2, Snf1-related protein kinase 2; YEL, yeast elicitor; YFP, yellow fluorescent protein.

Introduction

Stomata, formed by pairs of guard cells, serve as a major gateway for gas influx to and efflux from plants and microbe invasion, thus playing critical roles in plant growth and stress response. Guard cells regulate stomatal apertures in response to a variety of abiotic and biotic stimuli, such as light, drought and microbe infection (Israelsson et al. 2006, Shimazaki et al. 2007, Melotto et al. 2008). Elicitors derived from microbes are perceived by guard cells, resulting in stomatal closure to prevent microbe invasion (Melotto et al. 2008, Khokon et al. 2010). An elicitor derived from yeast (YEL) induces stomatal closure in Arabidopsis (Klüsener et al. 2002, Khokon et al. 2010, Salam et al. 2012, Ye et al. 2013).

Cytosolic Ca^{2+} has been recognized as a conserved second messenger in stomatal closure (Roelfsema and Hedrich 2010, Hubbard et al. 2012). In Arabidopsis, YEL triggers production of H_2O_2 mediated by salicylhydroxamic acid-sensitive peroxidases in rosette leaves and induces accumulation of H_2O_2 in guard cells (Khokon et al. 2010), and subsequently H_2O_2 activates non-selective Ca^{2+}-permeable cation (I_{Ca}) channels, leading to elevation of the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Klüsener et al. 2002, Khokon et al. 2010, Salam et al. 2012, Ye et al. 2013). Elevation of $[Ca^{2+}]_{cyt}$ is essential for activation of S-type anion channels, which is a hallmark of stomatal closure (Schroeder and Hagiwara 1989, Mori et al. 2006, Munemasa et al. 2007, Koers et al. 2011, Xue et al. 2011, Ye et al. 2013). Our recent studies revealed that a calcium-dependent protein kinase (CDPK), CPK6, positively functions in stomatal closure and activation of S-type anion channels in response to YEL (Ye et al. 2013).

An Snf1-related protein kinase 2 (SnRK2), Open Stomatal 1 (OST1), is a calcium-independent protein kinase and an essential positive regulator in ABA signaling in Arabidopsis guard cells. In resting conditions, OST1 kinase activity is inhibited by clade A type 2C protein phosphatases (PP2Cs). ABA promotes the interaction between PYR/PYL/RCAR (ABA receptors) and PP2Cs, resulting in reduction in PP2C activities and an increase in OST1 kinase activity (Cutler et al. 2010, Hubbard et al. 2010, Kollist et al. 2014). OST1 is involved not only in ABA-induced stomatal closure but also in stomatal closure induced by high CO_2, low humidity, ozone, darkness and a bacterial 22 amino acid, flagellin-derived peptide elicitor, flg22 (Melotto et al. 2006, Xie et al. 2006, Ache et al. 2010, Vahisalu et al. 2010, Xue et al. 2011, Merilo et al. 2013, Montillet et al. 2013). Recent studies showed that OST1, CPKs, complexes of calcineurin B-like proteins (CBLs) and CBL-interacting protein kinase, and GUARD CELL HYDROGEN PEROXIDE-RESISTANT1
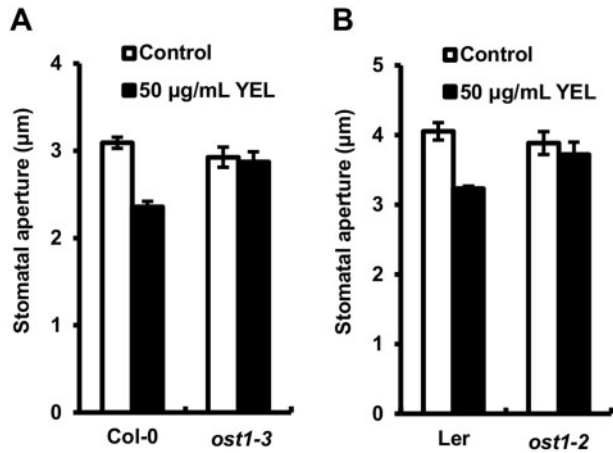
Fig. 1 YEL-induced stomatal closure. (A) YEL-induced closure in Col-0 and *ost1-3* plants. (B) YEL-induced stomatal closure in Ler and *ost1-2* plants. Averages from four independent experiments (100 total stomata per bar) are shown. Each error bar represents the SEM ($n = 4$).

(GHR1) can induce currents mediated by S-type anion channels, slow anion channel-associated 1 (SLAC1) and SLAH3 (SLAC1 homolog 3), in a heterologous expression system using *Xenopus* oocytes (Geiger et al. 2009, Lee et al. 2009, Geiger et al. 2010, Geiger et al. 2011, Brandt et al. 2012, Hua et al. 2012, Scherzer et al. 2012, Maierhofer et al. 2014). These results highlight that OST1 is essential for guard cell signaling, leading us to investigate whether OST1 is involved in YEL-induced stomatal closure.

In the present study, we investigated the role of OST1 in YEL-induced stomatal closure by using a knock-out mutant, *ost1-3*, and a kinase-deficient mutant, *ost1-2*. The presented results suggest that OST1 kinase is essential for stomatal closure and activation of S-type anion channels induced by YEL and that OST1 is not involved in H_2O_2 accumulation, I_{Ca} channel activation and $[Ca^{2+}]_{cyt}$ elevations in guard cells induced by YEL.

Results

Effects of *ost1* mutations on YEL-induced stomatal closure and activation of S-type anion currents

Application of $50\,\mu g\,ml^{-1}$ YEL induced stomatal closure in wild-type plants [$P < 0.001$ for Col-0 (**Fig. 1A**); $P < 0.001$ for Ler (**Fig. 1B**)] but not in *ost1* mutants [$P = 0.75$ for *ost1-3* (**Fig. 1A**); $P = 0.52$ for *ost1-2* (**Fig. 1B**)]. These results suggest that OST1 kinase is involved in YEL-induced stomatal closure.

Treatment with $50\,\mu g\,ml^{-1}$ YEL activated S-type anion currents in Col-0 guard cell protoplasts (GCPs) ($P < 0.0001$ at $-145\,mV$) (**Fig. 2A, B**) and in Ler GCPs (current $= -32.11 \pm 3.17\,pA$ at $-145\,mV$) (**Fig. 2E, F**). The YEL-activated S-type anion currents were impaired in the knock-out mutant *ost1-3* ($P < 0.01$ at $-145\,mV$) (**Fig. 2A–D**) and the kinase-deficient mutant *ost1-2* GCPs ($P < 0.01$ at $-145\,mV$) (**Fig. 2E–H**). These results suggest that kinase activity
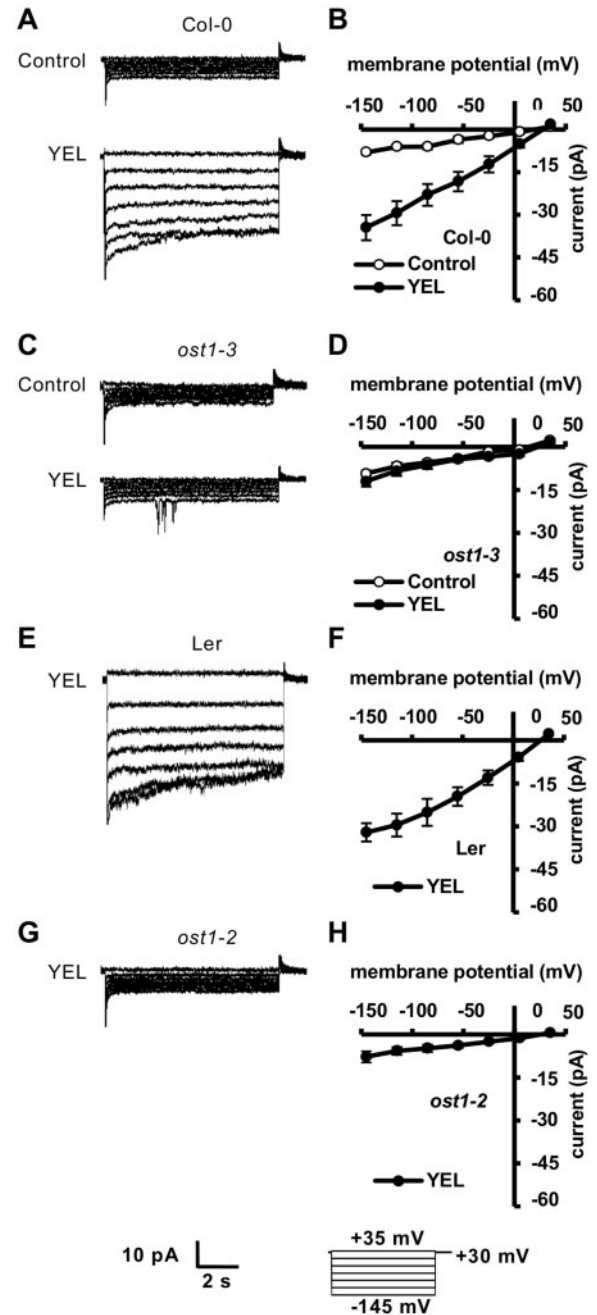

Fig. 2 YEL activation of S-type anion currents in GCPs. (A) S-type anion currents in Col-0 GCPs treated without (top trace) or with $50\,\mu g\,ml^{-1}$ YEL (bottom trace). (B) Steady-state current–voltage relationship of S-type anion currents as recorded in A (open circles, control; filled circles, YEL). (C) S-type anion currents in *ost1-3* GCPs treated without (top trace) or with $50\,\mu g\,ml^{-1}$ YEL (bottom trace). (D) Steady-state current–voltage relationship of S-type anion currents as recorded in C (open circles, control; filled circles, YEL). (E) S-type anion currents in Ler GCPs treated with $50\,\mu g\,ml^{-1}$ YEL. (F) Steady-state current–voltage relationship of S-type anion currents as recorded in E (filled circles, YEL). (G) S-type anion currents in *ost1-2* GCPs treated with $50\,\mu g\,ml^{-1}$ YEL. (H) Steady-state current–voltage relationship of S-type anion currents as recorded in G (filled circles, YEL). The voltage protocol was stepped up from $+35$ to $-145\,mV$ in $30\,mV$ decrements (holding potential, $+30\,mV$). GCPs were treated with YEL for 2 h before recordings. Each data point was obtained from at least five GCPs. Error bars represent the SE.
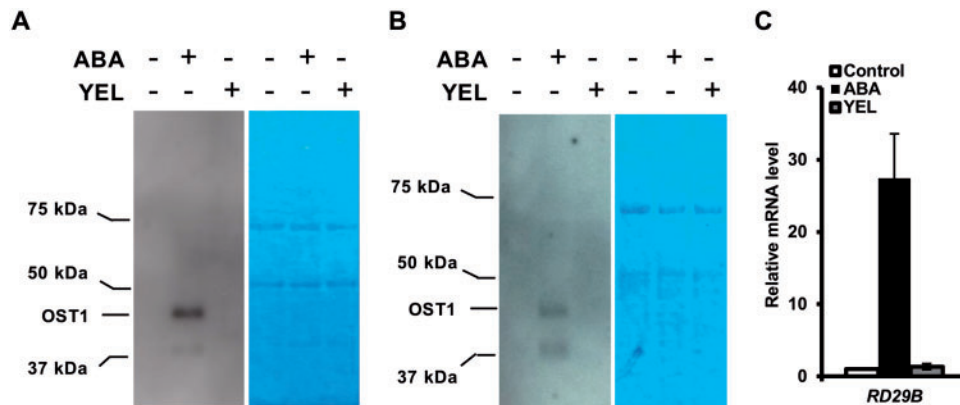
Fig. 3 Kinase activity of OST1 and transcript level of an ABA-responsive gene, *RD29B*, in GCPs. (A) Kinase activities of OST1 in Col-0 GCPs treated with 10 µM ABA and 50 µg ml^{-1} YEL (left, autoradiography; right, Coomassie blue staining). (B) Kinase activities of OST1 in Ler GCPs treated with 10 µM ABA and 50 µg ml^{-1} YEL (left, autoradiography; right, Coomassie blue staining). (C) Transcript levels of *RD29B* in Col-0 GCPs treated with 10 µM ABA and 50 µg ml^{-1} YEL.

of OST1 is required for activation of guard cell S-type anion channels by YEL.

Effects of YEL on kinase activity of OST1 and transcript level of RD29B in GCPs

The in-gel kinase assay using proteins from Col-0 and Ler GCPs treated with 10 µM ABA detected two radioactive signals, which correspond to OST1 and other SnRK2s (**Fig. 3A, B**; Boudsocq et al. 2007, Park et al. 2009, Fujii et al. 2009, Vlad et al. 2010, Kim et al. 2011), whereas the assay using GCPs treated with 50 µg ml^{-1} YEL did not detect either signal (**Fig. 3A, B**). The radioactive signals induced by ABA were abolished by mutations of *ost1-3* and *ost1-2* in GCPs (**Supplementary Fig. S1**). These results indicate that YEL does not increase the kinase activity of OST1 or other SnRK2s in Arabidopsis guard cells.

To confirm that YEL does not elevate the ABA content, we investigated the effect of YEL on the transcript level of an ABA-responsive gene, *Responsive to Desiccation 29B* (*RD29B*). In Col-0 GCPs, the transcript level of *RD29B* was increased by treatment with 10 µM ABA ($P < 0.01$) but not by treatment with 50 µg ml^{-1} YEL ($P = 0.46$) (**Fig. 3C**). These results show that YEL does not activate ABA signaling in guard cells.

Effects of *ost1* mutations on YEL-induced H₂O₂ accumulation, activation of I$_{Ca}$ currents and transient [Ca^{2+}]$_{cyt}$ elevations in guard cells

To clarify that OST1 is not involved in recruitment of second messengers in YEL signaling, we investigated YEL-induced H$_2$O$_2$ accumulation, activation of I$_{Ca}$ channels and transient [Ca^{2+}]$_{cyt}$ elevations in the *ost1* mutant guard cells.

Application of 50 µg ml^{-1} YEL significantly induced H$_2$O$_2$ accumulation in guard cells of Col-0 ($P < 0.01$; **Fig. 4A**), *ost1-3* ($P < 0.05$; **Fig. 4A**), Ler ($P < 0.05$; **Fig. 4B**) and *ost1-2* ($P < 0.01$; **Fig. 4B**). These results suggest that OST1 is not involved in YEL-induced H$_2$O$_2$ accumulation in guard cells.

Application of 50 µg ml^{-1} YEL activated I$_{Ca}$ currents in Col-0 GCPs ($P < 0.01$ at −180 mV) (**Fig. 5A, C**), *ost1-3* GCPs ($P < 0.01$ at −180 mV) (**Fig. 5B, D**), Ler GCPs ($P < 0.05$ at −180 mV) (**Fig. 5E**) and *ost1-2* GCPs ($P < 0.05$ at −180 mV) (**Fig. 5F**). These results indicate that OST1 is not involved in activation of I$_{Ca}$ channels by YEL in Arabidopsis guard cells.

When Col-0 epidermal tissues were not treated with YEL, 42.0% of Col-0 guard cells showed transient [Ca^{2+}]$_{cyt}$ elevations (**Supplementary Fig. S2**). Application of 50 µg ml^{-1} YEL induced transient [Ca^{2+}]$_{cyt}$ elevations in 95.4% of Col-0 guard cells (**Fig. 6A, C**). In untreated *ost1-3* epidermal tissues, 42.8% of guard cells showed transient [Ca^{2+}]$_{cyt}$ elevations (**Supplementary Fig. S2**). Application of 50 µg ml^{-1} YEL induced [Ca^{2+}]$_{cyt}$ elevations in 95.3% of *ost1-3* guard cells (**Fig. 6B, C**). There is no significant difference in the percentage of guard cells showing transient [Ca^{2+}]$_{cyt}$ elevations between Col-0 and *ost1-3* plants ($P = 0.97$). These results show that OST1 is not involved in YEL-induced transient [Ca^{2+}]$_{cyt}$ elevations in guard cells.

Effect of *abi1-1C* mutation on YEL-induced stomatal closure and activation of S-type anion currents

Gain-of-function mutation of a PP2C gene, *ABA INSENSITIVE 1* (*ABI1*) abolishes the ABA-induced OST1 kinase activity and constitutively inactivates OST1 kinase (Mustilli et al. 2002, Umezawa et al. 2009). To show that kinase activity of OST1 is involved in YEL-induced stomatal closure and activation of S-type anion channels, we investigated the effect of a gain-of-function mutation, *abi1-1C*, on stomatal closure and activation of S-type anion channels in response to YEL. Application of 50 µg ml^{-1} YEL did not induce stomatal closure in *abi1-1C* plants ($P = 0.75$; **Fig. 7A**). The YEL-activated S-type anion currents were impaired in the *abi1-1C* GCPs ($P < 0.01$ at −145 mV) (**Figs. 2A, B, 7B, C**). These results are consistent with the finding that kinase activity of OST1 is required for stomatal closure and activation of S-type anion channels in response to YEL.
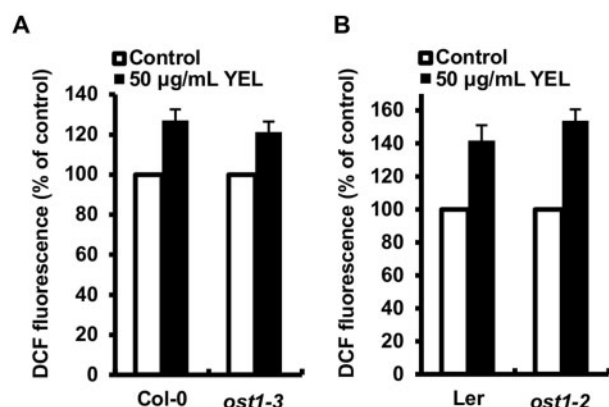
Fig. 4 YEL-induced H_2O_2 accumulation in guard cells. (A) H_2O_2 accumulation induced by 50 µg ml^{-1} YEL in Col-0 and *ost1-3* guard cells. (B) H_2O_2 accumulation induced by 50 µg ml^{-1} YEL in Ler and *ost1-2* guard cells. H_2O_2 accumulation was expressed as a percentage of the 2′,7′-dichlorofluorescein (DCF) fluorescence levels. Averages from three independent experiments (>150 total guard cells per bar in total) are shown.

Discussion

Plants perceive non-self elicitors characteristic of microbes, leading to the activation of the innate immune system. Deficiency in the innate immune system increases the disease susceptibility of host plants to adapted pathogens and even renders plants hosts for non-adapted pathogens. On the other hand, the innate immune system activated by perception of elicitors in the surface of cells is sufficient to ward off invasion of most microbes (Jones and Dangl 2006, Boller and Felix 2009, Macho and Zipfel 2014). Recent studies highlight that stomatal closure induced by elicitors is an innate immune response to prevent microbe infection (Melotto et al. 2008, McLachlan et al. 2014). Yeast elicitor extracted by ethanol precipitation mainly contains the fungal cell wall fraction and has been widely used as an elicitor to induce plant immune response including stomatal closure (Hahn et al. 1978, Schumacher et al. 1987, Gundlach et al. 1992, Blechert et al. 1995, Kollar et al. 1997, Klüsener et al. 2002, Ge and Wu 2005, Khokon et al. 2010, Salam et al. 2012, Ye et al. 2013). In the present study, we closely scrutinized the YEL-induced stomatal closure. The finding that the Ca^{2+}-independent kinase, OST1, is involved in stomatal closure induced by the biotic stimulus, YEL, leads us to propose a new model for how Ca^{2+}-dependent and Ca^{2+}-independent pathways co-ordinate stomatal closure induced by biotic and abiotic stimuli (see the following discussion).

OST1 kinase is essential for activation of S-type anion channels and stomatal closure in response to YEL

The protein kinase OST1 functions positively in stomatal closure induced by stimuli such as ABA, CO_2, low humidity, darkness, O_3 and flg22 (Melotto et al. 2006, Xie et al. 2006, Ache et al. 2010, Vahisalu et al. 2010, Xue et al. 2011, Merilo et al. 2013, Montillet et al. 2013). Involvement of OST1 in activation of

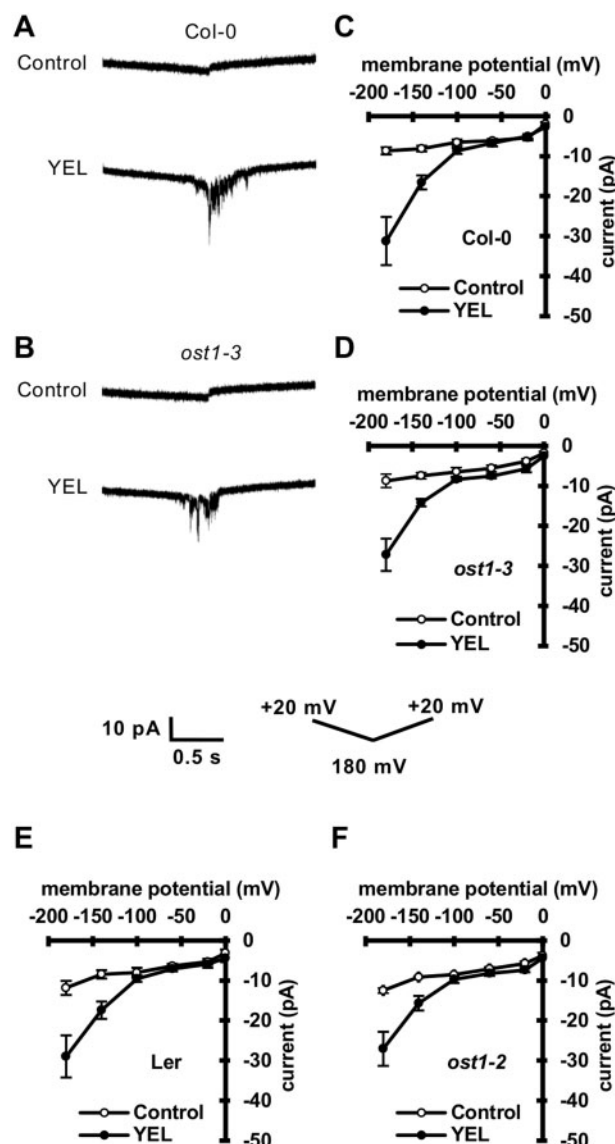**Fig. 5** YEL activation of I_{Ca} currents in GCPs. (A) I_{Ca} currents in Col-0 GCPs treated without YEL (top trace) or with 50 µg ml^{-1} YEL (bottom trace). (B) I_{Ca} currents in *ost1-3* GCPs treated without YEL (top trace) or with 50 µg ml^{-1} YEL (bottom trace). (C) Current–voltage relationship of I_{Ca} currents as recorded in A ($n = 5$; open circles, control; filled circles, YEL). (D) Current–voltage relationship of I_{Ca} currents as recorded in B ($n = 5$; open circles, control; filled circles, YEL). (E) Current–voltage relationship of I_{Ca} currents in Ler GCPs treated without YEL or with 50 µg ml^{-1} YEL ($n = 4$; open circles, control; filled circles, YEL). (F) Current–voltage relationship of I_{Ca} currents in *ost1-2* GCPs treated without YEL or with 50 µg ml^{-1}L YEL ($n = 3$; open circles, control; filled circles, YEL). A ramp voltage protocol from +20 to −180 mV (holding potential, 0 mV; ramp speed, 200 mV s^{-1}) was used. After making the whole-cell configuration, GCPs were recorded 16 times to obtain averages for the control. After adding YEL extracellularly, the GCPs were recorded 16 times to obtain averages for the YEL treatment. The interpulse period was 1 min.

guard cell S-type anion channels by abiotic stimuli such as ABA and CO_2 has been studied (Xue et al. 2011, Acharya et al. 2013, Tian et al. 2015), but involvement of OST1 in activation by biotic stimuli remains to be clarified. The presented
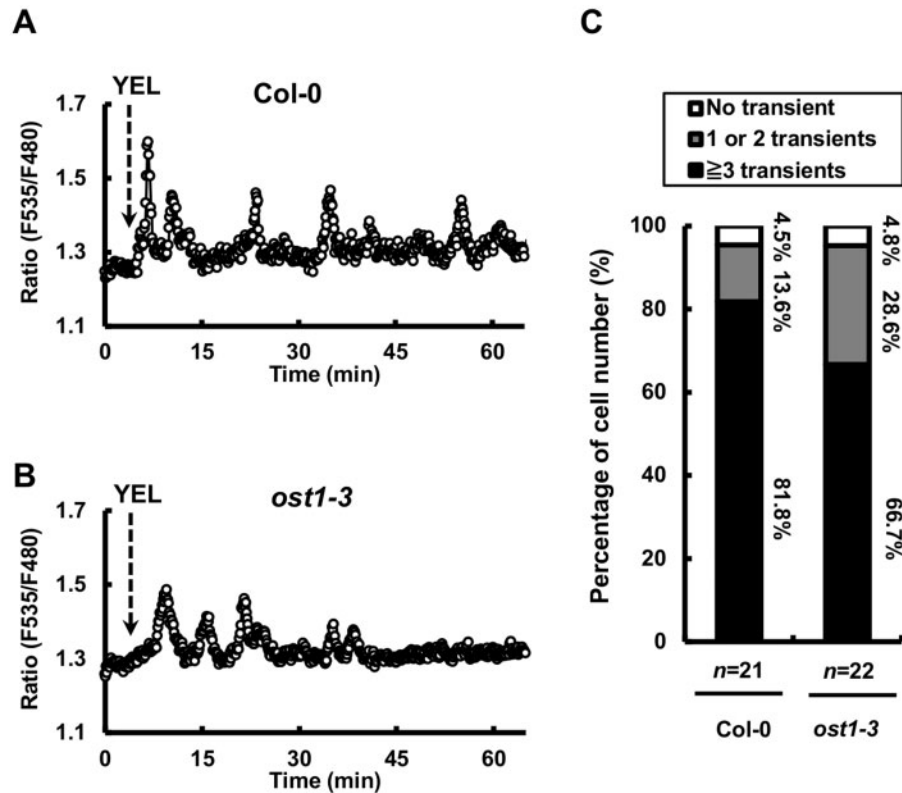
Fig. 6 YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations in Col-0 and *ost1-3* guard cells expressing the Ca^{2+}-sensing fluorescent protein YC 3.6. (A) A representative trace of fluorescence emission ratios (535/480 nm) showing 50 µg ml^{-1} YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations in Col-0 guard cells. (B) A representative trace of fluorescence emission ratios (535/480 nm) showing 50 µg ml^{-1} YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations in *ost1-3* guard cells. (C) Percentage of guard cells showing a different number of YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations in Col-0 and *ost1-3* guard cells. $[Ca^{2+}]_{cyt}$ elevations were counted when changes in fluorescence emission ratios were ≥ 0.1 from the baseline.
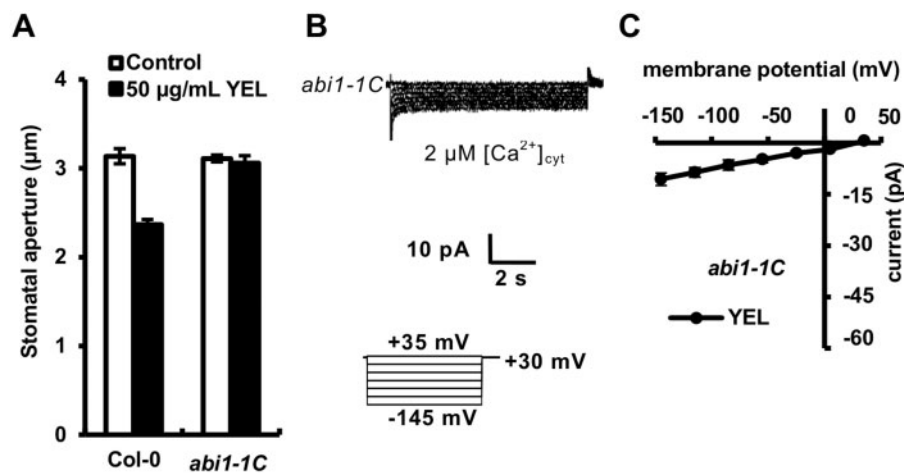

Fig. 7 YEL-induced stomatal closure and activation of S-type anion currents in GCPs in *abi1-1C* plants. (A) YEL-induced stomatal closure in Col-0 and *abi1-1C* plants. Averages from four independent experiments (100 total stomata per bar) are shown. Error bars represent SEs ($n = 4$). (B) S-type anion currents in *abi1-1C* GCPs at 2 µM $[Ca^{2+}]_{cyt}$ treated with 50 µg ml^{-1} YEL. (C) Steady-state current–voltage relationship of S-type anion currents as recorded in B (*abi1-1C*, $n = 4$; filled circles, YEL). The voltage protocol was stepped up from +35 to -145 mV in 30 mV decrements (holding potential, +30 mV). GCPs were treated with YEL for 2 h before recordings. Error bars represent SEs.

results provided in vivo evidences that OST1 kinase is involved in stomatal closure and activation of S-type anion channels induced by a biotic stimulus, YEL.

Though it is well known that OST1 is essential for stomatal closure in response to a variety of stimuli (Melotto et al. 2006, Xie et al. 2006, Ache et al. 2010, Vahisalu et al. 2010, Xue et al. 2011, Merilo et al. 2013, Montillet et al. 2013), to our knowledge there is no evidence that kinase activity of OST1 is increased in guard cells in response to stimuli other than ABA. On the other hand, it has been reported that flg22 does not elicit activation

of OST1 kinase in Arabidopsis suspension cells (Montillet et al. 2013). The present study shows that YEL did not increase OST1 kinase activity or trigger ABA signaling (**Fig. 3**), and our previous results show that endogenous ABA is not required for YEL-induced stomatal closure (Issak et al. 2013), suggesting that YEL does not increase OST1 kinase activity in Arabidopsis guard cells.

A recent paper has shown that a receptor kinase, Guard Cell Hydrogen Peroxide-Resistant 1 (GHR1), is required for activation of S-type anion channels and induction of stomatal closure by ABA and H_2O_2. In GHR1-expressed mesophyll protoplasts, GHR1 has basal kinase activity, which is not elevated by ABA or H_2O_2 (Hua et al. 2012). In one of 12 repetitions of in-gel kinase assays, we observed weak basal activity of OST1 kinase (**Supplementary Fig. S1B**, lane 1). Therefore, we propose that basal activity of OST1 kinase is involved in YEL signaling in guard cells.

OST1 is not involved in H_2O_2 accumulation, activation of I_{Ca} channels and transient $[Ca^{2+}]_{cyt}$ elevations in guard cells induced by YEL

H_2O_2 functions as an important second messenger during stomatal closure (Pei et al. 2000, Zhang et al. 2001, Khokon et al. 2011, Salam et al. 2012). In ABA signaling, two NADPH oxidases, AtrbohD and AtrbohF, catalyze superoxide production in the apoplasts, leading to H_2O_2 production through dismutation (Kwak et al. 2003, Jannat et al. 2011, Jannat et al. 2012). On the other hand, in YEL signaling, apoplastic peroxidases mainly mediate H_2O_2 production (Khokon et al. 2010). The produced H_2O_2 can accumulate in guard cells through diffusion and water channels (Henzler and Steudle 2000, Bienert et al. 2007). The OST1 kinase is essential for ABA-induced H_2O_2 accumulation in guard cells by regulating activation of AtrbohD and AtrbohF (Mustilli et al. 2002, Sirichandra et al. 2009, Acharya et al. 2013). On the other hand, YEL-induced H_2O_2 accumulation was not impaired in *ost1* mutants (**Fig. 4**), which suggests that OST1 is not involved in peroxidase-mediated H_2O_2 accumulation in response to YEL.

Previous studies showed that YEL activation of I_{Ca} channels requires CPK6 (Ye et al. 2013). In the present study, OST1 does not appear to be required for activation by YEL of I_{Ca} channels because YEL-induced I_{Ca} currents were not impaired in *ost1* guard cells (**Fig. 5**). Since activation by YEL of I_{Ca} channels is modulated by redox status (Klüsener et al. 2002, Khokon et al. 2010, Ye et al. 2013), the activation of I_{Ca} channels induced by YEL in *ost1* guard cells may be attributed to the H_2O_2 accumulation (**Figs. 4**, **5**).

In Arabidopsis, YEL-induced stomatal closure is accompanied by transient $[Ca^{2+}]_{cyt}$ elevations (Klüsener et al. 2002, Khokon et al. 2010, Salam et al. 2012, Ye et al. 2013). Transient $[Ca^{2+}]_{cyt}$ elevations are initiated by influx of Ca^{2+} through I_{Ca} channels from the apoplast in guard cells (Pei et al. 2000, Allen et al. 2001, Murata et al. 2001) and then the Ca^{2+} released from internal stores participates in the following transient elevations of $[Ca^{2+}]_{cyt}$ (Garcia-Mata et al. 2003, Lemtiri-Chlieh et al. 2003). In the present study, *ost1-3* mutation did not suppress YEL-induced $[Ca^{2+}]_{cyt}$ elevations (**Fig. 6**).

These results suggest that OST1 does not function in Ca^{2+} influx from the apoplast or Ca^{2+} release from internal stores induced by YEL. In addition, the transcription level of *CPK6* in the leaves of *ost1-3* plants is not significantly different from that of Col-0 plants (**Supplementary Fig. S3**). Taken together, these results indicate that OST1 is not involved in a Ca^{2+}-dependent pathway mediated by CPK6 in YEL-induced stomatal closure.

Integration of Ca^{2+}-dependent and Ca^{2+}-independent pathways in guard cell signaling

Both Ca^{2+}-dependent and Ca^{2+}-independent pathways regulate ABA-induced stomatal closure (Roelfsema and Hedrich 2010, Hubbard et al. 2012). For the Ca^{2+}-dependent pathway, CPKs have been identified as key regulators. However, the mechanism underlying the Ca^{2+}-independent pathway leading to ABA stomatal closure remains to be clarified. Increasing evidence implies that OST1 kinase, whose kinase activity is independent of Ca^{2+} (Mustilli et al. 2002), regulates the Ca^{2+}-independent pathway in ABA signaling, but in vivo evidence is difficult to obtain since OST1 kinase regulates the Ca^{2+}-dependent pathway induced by ABA in guard cells (Mustilli et al. 2002, Acharya et al. 2013).

Biotic elicitors also induce stomatal closure through the Ca^{2+}-dependent pathway (Lee et al. 1999, Klüsener et al. 2002, Ye et al. 2013). The presented study shows that OST1 kinase is essential for YEL-induced stomatal closure, while OST1 is not essential for YEL-induced $[Ca^{2+}]_{cyt}$ elevations in guard cells, thus providing in vivo evidence that OST1 kinase mediates a Ca^{2+}-independent pathway in stomatal closure induced by a biotic stimulus, YEL.

Activation of S-type anion channels requires not only $[Ca^{2+}]_{cyt}$ elevation but also priming by ABA and CO_2 (Schroeder and Hagiwara 1989, Allen et al. 2002, Siegel et al. 2009, Xue et al. 2011). In the *ost1* mutants, YEL did not activate S-type anion channels even when $[Ca^{2+}]_{cyt}$ was elevated. These results suggest that the OST1 with basal kinase activity puts S-type anion channels on standby to be activated by CPK6 (**Fig. 8**).

Based on the presented results and previous studies, we propose a model to integrate Ca^{2+}-dependent and Ca^{2+}-independent pathways in guard cell signaling as follows: S-type anion channels are phosphorylated by OST1 with basal kinase activity to stand by for phosphorylation by CPKs. Stimuli such as ABA and YEL induce activation of CPKs via the Ca^{2+}-dependent pathway, leading to S-type anion channel activation and stomatal closure.

Materials and Methods

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) wild-type Columbia (Col-0), Landsberg erecta (Ler), *ost1-2* (Mustilli et al. 2002), *ost1-3* (Yoshida et al. 2002) and *abi1-1C* (Col-0; Merilo et al. 2013) were grown in pots containing a mixture of 70% (v/v) vermiculite (Asahi-kogyo) and 30% (v/v) kureha soil (Kuhera chemical) in a growth chamber (photon flux density of 80 µmol m^{-2} s^{-1} under a 16 h light/8 h dark regime). The temperature and relative humidity in the growth chamber were 22 ± 2°C and 60 ± 10%. Twice or three times a week, 0.1% Hyponex
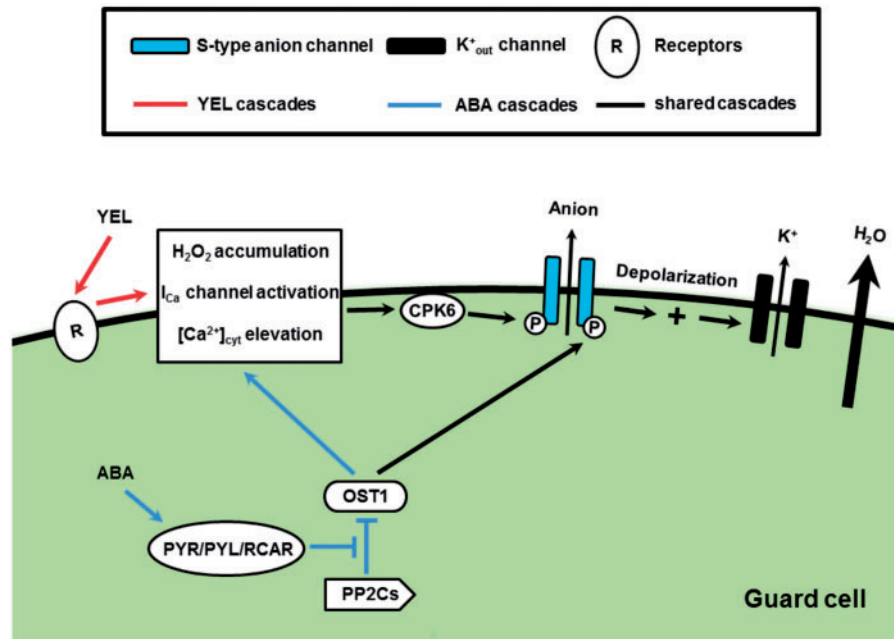
Fig. 8 A proposed model for YEL signaling in guard cells. OST1 with basal kinase activity phosphorylates S-type anion channels to make them stand by to be activated by CPK6. Once YEL is perceived by certain receptors located in the plasma membrane of guard cells, YEL signal is transformed to CPK6 activation via H_2O_2 accumulation, I_{Ca} channel activation and $[Ca^{2+}]_{cyt}$ elevation. On the other hand, ABA is perceived by PYR/PYL/RCAR, leading to inhibition of PP2Cs and an increase in OST1 kinase activity. This OST1 kinase activation is transformed to CPK6 activation via H_2O_2 accumulation, I_{Ca} channel activation and $[Ca^{2+}]_{cyt}$ elevation. The CPK6 phosphorylates S-type anion channels that are standing by for phosphorylation by CPK6. This phosphorylation leads to activation of S-type anion channels, which sequentially induces depolarization of plasma membrane, activation of outward K^+ channels (K^+_{out} channels), water efflux and stomatal closure.

solution was provided to the plants. $[Ca^{2+}]_{cyt}$ in guard cells was measured using a Ca^{2+}-sensing fluorescent protein, YC3.6 (Nagai et al. 2004). To obtain YC3.6-expressing *ost1-3* mutants, *ost1-3* mutants were crossed with Col-0 plants that had previously been transformed with YC3.6. Homozygous plants of the *ost1-3* mutant expressing YC3.6 were used for experiments. All the plants used for experiments were 4–6 weeks old.

Elicitor preparation

YEL was prepared as described previously (Schumacher et al. 1987). Briefly, 200 g of commercial baker's yeast was dissolved in 300 ml of 20 mM sodium citrate buffer (pH 7.0) and autoclaved at 121°C and 110,000 Pa for 60 min. The autoclaved suspension was centrifuged at $10,000 \times g$ for 20 min and the supernatant and ethanol were mixed in equal volume and stirred gently overnight. The mixture was centrifuged at $10,000 \times g$ for 20 min and the supernatant was mixed with three times its volume of ethanol and stirred gently overnight. The precipitate obtained by decantation was lyophilized and stored at $-80°C$ until use.

Stomatal aperture measurement

Fully expanded rosette leaves were excised for stomatal aperture measurements as described previously (Uraji et al. 2012). Leaves were floated on the assay solution in the light for 2 h to open the stomata. Then YEL (50 µg ml^{-1}) was added, and the leaves were kept in the light for 2 h before measurement. For measurement of stomatal apertures, the leaves were shredded for 30 s, and epidermal tissues were collected using nylon mesh. Thirty stomatal apertures were measured for each sample.

Patch-clamp measurement

Current measurements of I_{Ca} and S-type anion channels in Arabidopsis guard cells were performed as described previously (Ye et al. 2013). Arabidopsis GCPs were prepared from rosette leaves. For I_{Ca} current measurement, pipette solution contained 10 mM BaCl$_2$, 0.1 mM dithiothreitol (DTT), 5 mM NADPH, 4 mM EGTA 10 mM HEPES-Tris, pH 7.1. Bath solution contained 100 mM

BaCl$_2$, 0.1 mM DTT and 10 mM MES-Tris, pH 5.6. A ramp voltage protocol from +20 to −180 mV (holding potential, 0 mV; ramp speed, 200 mV s^{-1}) was used. For S-type anion current measurement, pipette solution contained 150 mM CsCl, 2 mM MgCl$_2$, 6.7 mM EGTA, 5.8 mM CaCl$_2$ ($[Ca^{2+}]_{cyt}$, 2 µM), 5 mM ATP and 10 mM HEPES-Tris, pH 7.1. Bath solution contained 30 mM CsCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$ and 10 mM MES-Tris, pH 5.6. The voltage protocol was stepped up from +35 to −145 mV in 30 mV decrements (holding potential, +30 mV). In all cases, osmolarity was adjusted to 500 mmol/kg^{-1} (pipette solutions) and 485 mmol kg^{-1} (bath solutions) with D-sorbitol.

Imaging of $[Ca^{2+}]_{cyt}$ in guard cells

Col-0 and *ost1-3* plants expressing YC3.6 were used for the measurement of $[Ca^{2+}]_{cyt}$ in guard cells as described previously (Ye et al. 2013). The abaxial side of an excised leaf was gently mounted on a glass slide with a medical adhesive (stock no. 7730; Hollister) followed by removal of the adaxial epidermis and the mesophyll tissue with a razor blade in order to keep the lower epidermis intact on the slide. The remaining abaxial epidermis was incubated in solution containing 5 mM KCl, 50 µM CaCl$_2$ and 10 mM MES-Tris (pH 6.15) in the light for 2 h at 22°C to promote stomatal opening. Turgid guard cells were used to measure $[Ca^{2+}]_{cyt}$. The observation chamber was perfused with the solution using a peristaltic pump. Guard cells were incubated in the absence of YEL for 5 min and then in the presence of YEL. For dual-emission ratio imaging of YC3.6, we used a 440AF21 excitation filter, a 445DRLP dichroic mirror, a 480AF30 emission filter for cyan fluorescent protein (CFP) and a 535DF25 emission filter for yellow fluorescent protein (YFP). The CFP and YFP fluorescence intensity of guard cells was imaged and analyzed using the W-View system and AQUA COSMOS software (Hamamatsu Photonics). CFP and YFP fluorescence was monitored simultaneously.

Detection of H_2O_2 in guard cells

H_2O_2 accumulation in guard cells was evaluated using 2′,7′-dichlorodihydro-fluorescein diacetate (H$_2$DCF-DA) (Ye et al. 2013). Epidermal tissues were isolated after rosette leaves were shredded with a commercial blender.

The epidermal tissues were incubated in medium containing 5 mM KCl, 50 μM CaCl$_2$ and 10 mM MES-Tris, pH 6.15, in the light at room temperature for 3 h. After incubation, 50 μM H$_2$DCF-DA was added to the medium. The epidermal tissues were incubated in the dark at room temperature for 30 min, and then the excess dye was washed out with the same medium. The dye-loaded tissues were treated with 50 μg ml^{-1} YEL in the dark at room temperature for 20 min. The stained guard cells were imaged using a fluorescence microscope (Biozero BZ-8000, Keyence) with an OP-66835 BZ GFP filter (excitation wavelength, 480/30 nm; absorption wavelength, 510 nm (long-pass); and dichroic mirror wavelength, 505 nm). Fluorescence pictures of the guard cells were analyzed by Image J software (NIH), and expressed as a percentage of the control.

RNA extraction and real-time PCR

A large amount of GCPs (5×10^6 cells for each sample) was isolated as described previously (Ueno et al. 2005). Isolated GCPs were incubated in solution containing 1 mM CaCl$_2$, 0.4 mM mannitol and 10 mM MES-KOH (pH 5.4), supplemented with 10 μM ABA or 50 μg ml^{-1} YEL in the light for 2 h. RNA was then extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 500 ng of RNA using PrimeScript RT Master Mix (Perfect Real Time) (TAKARA BIO INC.) according to the manufacturer's instructions. Quantitative real-time PCR was performed with an Mx3000P QPCR System (Agilent Technologies) using SYBR Green (Brilliant II QPCR Master Mix; Stratagene) to monitor double-stranded DNA synthesis. The levels of gene transcript were normalized to that of TUB2 and expressed relative to the amounts observed. Primers used in PCR amplification are as follows: for RD29B (At5g52300), 5'-CGAGCAAGACCCAGAAGTTCAC-3' and 5'-TTACCCGTTACACCACCTCTCA-3'; for TUB2 (At3g18780), 5'-AAACTCACTACCCCCAGCTTTG-3' and 5'-CACCAGACATAGTAGCAGAAATCAAGT-3'. The experiment was repeated four times.

In-gel kinase assay

In-gel kinase analyses were performed as described previously (Kim et al. 2011). A large amount of GCPs (5×10^6 cells for each sample) were isolated as described previously (Ueno et al. 2005). Isolated GCPs were incubated in solution containing 1 mM CaCl$_2$, 0.4 mM mannitol and 10 mM MES-KOH (pH 5.4), supplemented with 10 μM ABA or 50 μg ml^{-1} YEL in the light for 30 min. Protein was extracted from GCPs using extraction buffer containing 100 mM HEPES, pH 7.6, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 150 mM NaCl, 500 μM DTT, 10 mM NaF, 5 mM Na$_3$VO$_4$, 5 mM β-glycerophosphate, 1/200 vol. of protease inhibitor (P9599, Sigma-Aldrich) and 1/200 vol. of phosphatase inhibitor (P0044, Sigma-Aldrich). For electrophoresis, each protein sample was loaded at 10 μg per lane to a 10% polyacrylamide gel that contained SDS and 0.25 mg ml^{-1} histone III-S. After electrophoresis, the gel was washed three times with washing buffer (25 mM Tris–HCl pH 8.0, 0.5 mM DTT, 0.1 mM Na$_3$VO$_4$, 5 mM NaF, 0.5 mg ml^{-1} bovine serum albumin and 0.1% Triton X-100) for 30 min at room temperature, followed by two washes with renaturation buffer (25 mM Tris–HCl pH 8.0, 1 mM DTT, 0.1 mM Na$_3$VO$_4$ and 5 mM NaF) for 30 min at room temperature and one wash at 4°C overnight. The gel was equilibrated with reaction buffer (25 mM HEPES pH 7.5, 2 mM EGTA, 12 mM MgCl$_2$, 1 mm DTT, 0.1 mM Na$_3$VO$_4$) for 30 min at room temperature and then incubated in 20 ml of reaction buffer with 1.85 MBq of [γ-^{32}P]ATP for 90 min at room temperature. The gel was washed with washing solution (5% trichloroacetic acid, 1% pyrophosphoric acid) until no more radioactivity was detected in the washing solution. The washed gels were then stained with Coomassie Brilliant Blue and dried on filter paper. The radioactivity of the gels was detected with X-ray film.

Statistical analysis

The significance of differences between data sets was assessed by Student's t-test. The response of $[Ca^{2+}]_{cyt}$ was assessed by χ^2 test. Differences were considered significant for $P < 0.05$.

Accession numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: OST1 (AT4G33950), RD29B (At5g52300), TUB2 (AT5G62690) and ABI1 (AT4G26080).

Supplementary data

Supplementary data are available at PCP online.

Disclosures

The authors have no conflicts of interest to declare.

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